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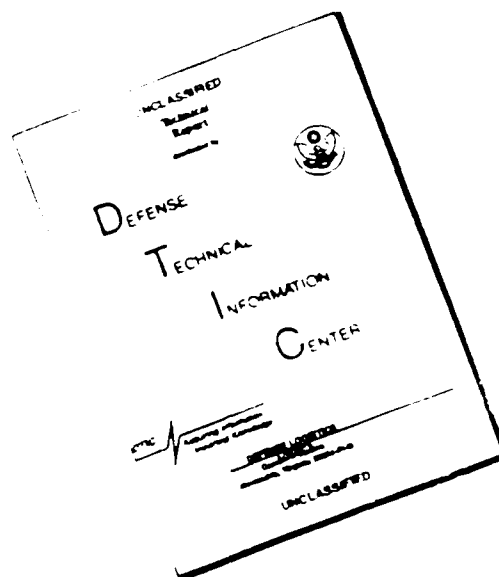
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2. REPORT DATE 1993		3. REPORT TYPE AND DATES COVERED Journal article	
4. TITLE AND SUBTITLE Expression and diagnostic utility of hepatitis E virus putative structural proteins expressed in insect cells		5. FUNDING NUMBERS PE - 62787A PR - 3M162787.A870 TA - A2 WU -1287	
6. AUTHOR(S) He J; Tam AW; Yarbough PO; Reyes GR; Carl M			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Medical Research Institute Commanding Officer 8901 Wisconsin Avenue Bethesda, Maryland 20889-5607		8. PERFORMING ORGANIZATION REPORT NUMBER  NMRI 93-70	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Naval Medical Research and Development Command National Naval Medical Center Building 1, Tower 12 8901 Wisconsin Avenue Bethesda, Maryland 20889-5606		10. SPONSORING/MONITORING AGENCY REPORT NUMBER  DN243534	
11. SUPPLEMENTARY NOTES Reprinted from: Journal of Clinical Microbiology 1993 Aug; Vol.31 No.8 pp. 2167-2173			
12a. DISTRIBUTION/AVAILABILITY STATEMENT  Approved for public release; distribution is unlimited.		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  <div style="text-align: center;"><b>DTIC</b> <b>SELECTE</b> <b>DEC 07 1993</b> <b>S B D</b></div>			
14. SUBJECT TERMS Hepatitis E; Virology; Baculovirus; Recombinant proteins; Diagnostic tests		15. NUMBER OF PAGES 7	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT  Unlimited

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## Expression and Diagnostic Utility of Hepatitis E Virus Putative Structural Proteins Expressed in Insect Cells

JUNKUN HE,<sup>1</sup> ALBERT W. TAM,<sup>2</sup> PATRICE O. YARBOUGH,<sup>2</sup>  
GREGORY R. REYES,<sup>2†</sup> AND MITCHELL CARL<sup>1\*</sup>

*Accelerated Product Development Program, Naval Medical Research Institute,  
Bethesda, Maryland 20889-5055,<sup>1</sup> and Molecular Virology Department,  
Genelabs Inc., Redwood City, California 94063<sup>2</sup>*

Received 14 January 1993/Accepted 4 May 1993

The full-length putative structural proteins encoded by open reading frame 2 (ORF2) and ORF3 of hepatitis E virus have been cloned and expressed in recombinant baculovirus. Sera obtained from 28 Sudanese pediatric patients with acute hepatitis and 19 pediatric control patients were analyzed for reactivity to hepatitis E virus by using the baculovirus-expressed ORF2 and ORF3 proteins in a Western blot (immunoblot) format. Seventeen of the 18 patients classified as having non-A, non-B hepatitis, without acute antibody markers for hepatitis A, B, or C viruses, Epstein-Barr virus, or cytomegalovirus, were shown to have immunoglobulin M (IgM) antibodies to the recombinant ORF2 protein, as did two patients with chronic hepatitis B, three of seven patients with acute hepatitis A, and one patient with acute hepatitis B. None of the 19 control patients had IgM antibodies against the ORF2 or ORF3 proteins. The Western blot assay using the baculovirus-expressed ORF3 protein did not appear to be as sensitive as the assay based on the ORF2 protein. Only 10 of the patients classified as having non-A, non-B hepatitis had IgM antibodies to the baculovirus-expressed ORF3 protein. We conclude that a Western blot assay which uses a baculovirus-expressed ORF2 protein is both sensitive and specific for diagnosing acute hepatitis E.

Enterically transmitted non-A, non-B (ET-NANB) hepatitis occurs in epidemics and sporadically in many geographic locations, including India (12, 13, 25, 32), Nepal (10), Burma (18, 24), Pakistan (4), Mexico (28), the former Soviet Union (1), Africa (2, 23), North America (5), and western Europe (17). ET-NANB hepatitis was first documented in New Delhi, India, in 1955 (30) and is primarily associated with the ingestion of fecally contaminated drinking water. Acute outbreaks of ET-NANB hepatitis primarily affect young to middle-aged adults and are often associated with a high mortality rate in infected pregnant women, approaching 20% in many reported epidemics (19). Hepatitis E disease is transient and self-resolving and is similar to hepatitis A clinically and epidemiologically except for the high mortality rate in pregnant women. Recently, an ET-NANB hepatitis agent has been cloned and sequenced and has been termed hepatitis E virus (HEV) (21, 27). The virus appears to be a polyadenylated, positive-stranded RNA virus with two major open reading frames (ORFs). The largest ORF (ORF1) is believed to encode nonstructural proteins such as the RNA-dependent RNA polymerase (21), a putative helicase (6), and others recognized from structural motifs (15). The second major ORF (ORF2) and the small third ORF (ORF3) have been shown to encode highly immunogenic antigens and may encode the structural proteins of HEV (27, 33). Diagnostic assays using ORF3 or truncated ORF2 recombinant HEV structural proteins fused to Sj26 or to *trpE* and expressed in *Escherichia coli* have been described recently (3, 7, 20, 33). In addition, an immunofluorescence assay using primate liver tissue infected with HEV has also been described (16).

In this article, we report on the expression of the full-

length HEV proteins encoded by ORF2 and ORF3 produced by recombinant baculovirus. Both antigens were used in a Western blot (immunoblot) assay in order to diagnose hepatitis E in pediatric patients presenting with acute hepatitis in the Sudan. Although 17 of 18 patients with NANB hepatitis had immunoglobulin M (IgM) antibodies that were reactive with the ORF2-encoded protein, only 10 of the 18 patients had IgM antibodies reactive with the ORF3-encoded protein. The Western blot assay based on the ORF2-encoded protein appears to be both sensitive and specific for the diagnosis of acute hepatitis E.

### MATERIALS AND METHODS

**Construction of recombinant transfer plasmids.** The plasmid BET8 (26), which contains the entire HEV (Burma strain) ORF3, and a recombinant  $\lambda$ gt10 bacteriophage (BET1), which contains all of ORF2 except for 19 nucleotides at the 5' end (26), were used as templates in the polymerase chain reaction (PCR) (24), which resulted in the entire ORF2 and ORF3 amplified DNA fragments with flanking *Xba*I restriction endonuclease cleavage sites. Thirty-five PCR cycles were performed in a 50- $\mu$ l volume containing each of the two primers at 0.1  $\mu$ M. PCR buffer (U.S. Biochemicals, Cleveland, Ohio), 25 mM MgCl<sub>2</sub>, and 1 U of *Taq* polymerase (U.S. Biochemicals). Each cycle consisted of a denaturation at 95°C for 45 s, primer annealing for 1 min at 37°C, and primer extension at 72°C for 3 min. Primers used to amplify ORF2 were primer 1, 5'-GCG-CGC-TCT-AGA-ATG-CGC-CGG-CTT-CCT-ATT-TTG-TTG-CTG-CTC-CTC-ATG-TTT-TTG-CCT-ATG-CTG-CCC-3', corresponding to 54 bases from nucleotide 5146 to 5200, and primer 2, 5'-GCG-CGC-TCT-AGA-CTA-CTA-CAA-CTC-CCG-AGT-TTT-ACC-CAC-CTT-CAT-CTT-AAG-GCG-CTG-AAG-CTC-AGC-GACAGT-3', corresponding to 57 bases from 7072 to 7129 (27). Primer 3, 5'-GCG-CGC-TCT-AGA-ATG-

\* Corresponding author.

† Present address: Triplex Pharmaceutical Corporation, The Woodlands, TX 77380.

AAT-AAC-ATG-TCT-TTT-GCT-GCG-CCC-3', corresponding to 29 bases from 5105 to 5153, and primer 4, 5'-GCG-GCG-TCT-AGA-TTA-TTA-GCG-GCG-CCC-CAG-CTG-TGG-3', which corresponds to 24 bases from 5454 to 5478 (27), were used to amplify ORF3. Each primer also contained flanking *Xba*I restriction endonuclease cleavage sites (underlined) and 6 additional nucleotides at the 5' end. The ORF2 and ORF3 PCR products were digested with *Xba*I (New England Biolabs, Beverly, Mass.) and ligated into the compatible *Nhe*I site in transfer vector pBlue-Bac2 (Invitrogen, San Diego, Calif.) which was previously digested with *Nhe*I and treated with alkaline phosphatase (Boehringer-Mannheim, Indianapolis, Ind.). After transformation into INV $\alpha$  competent cells, ampicillin-resistant colonies were screened for the presence of the appropriate recombinant plasmids (22). The orientation of the inserted fragments in pBlue-Bac2 was confirmed by restriction enzyme analysis and by partial DNA sequencing of the subcloned fragments by the method of Sanger (22). A recombinant plasmid containing the ORF2 fragment was digested with *Hind*III and a recombinant plasmid containing the ORF3 fragment was digested with *Bam*HI and *Nco*I (Bethesda Research Laboratories, Gaithersburg, Md.) in order to determine the correct orientation.

**Generation of recombinant baculovirus.** *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant AcNPV (rAcNPV) were grown in *Spodoptera frugiperda* 9 (Sf9) insect cells in Grace's medium (Invitrogen) supplemented with 10% fetal bovine serum. Recombinant transfer vector and wild-type AcNPV DNA were used to cotransfect Sf9 cells (Invitrogen) (31). Blue baculovirus recombinant (rAcNPV) plaques were then selected and expanded in Sf9 cells. Stocks of independent recombinant viruses were stored at 4°C.

**Protein preparation and immunoblot analysis.** For initial screening, 10<sup>6</sup> Sf9 cells were infected with recombinant virus (rAcNPV) at a multiplicity of infection of approximately 3 for ORF2-rAcNPV and approximately 2 for ORF3-rAcNPV and incubated at 27°C for 48 h. The cells were pelleted by centrifugation at 3,000  $\times$  g for 10 min. The supernatant was recovered and stored at 4°C. The cell pellets were suspended in 0.5 ml of 2% sodium dodecyl sulfate (SDS)-0.2 M NaCl-0.2 M Tris (pH 7.5)-1.5 mM MgCl<sub>2</sub>, heated to 100°C for 3 min, and stored at -20°C. These cell lysates (as well as identically treated uninfected Sf9 cells and Sf9 cells infected with wild-type baculovirus) and supernatants were then diluted with 2 $\times$  protein sample buffer and boiled for 10 min, and equal quantities of each protein were separated by SDS-12.5% polyacrylamide gel electrophoresis (PAGE).

Proteins separated by SDS-PAGE were electroblotted (22) onto nitrocellulose membranes in transfer buffer containing 25 mM Tris, 190 mM glycine, and 20% methanol. Membranes were blocked with phosphate-buffered saline (PBS) containing 1% sodium caseinate, 0.05% sodium azide, and 0.05% Tween 20 for 1 h at room temperature, and some membranes were cut into 2-mm strips. The nitrocellulose membranes were then incubated with optimal concentrations of patient sera (1:900 for the recombinant ORF2 protein and 1:300 for the recombinant ORF3 protein) for 2 h at 4°C. Preliminary experiments suggested that these concentrations resulted in a maximum signal with a minimum of background (data not shown). Sera were obtained from pediatric patients who presented with acute hepatitis in Khartoum, Sudan (9). After being washed four times with PBS containing 0.05% Tween and 0.05% sodium azide (wash medium), the strips were then incubated with a 1:1,000 dilution of goat alkaline

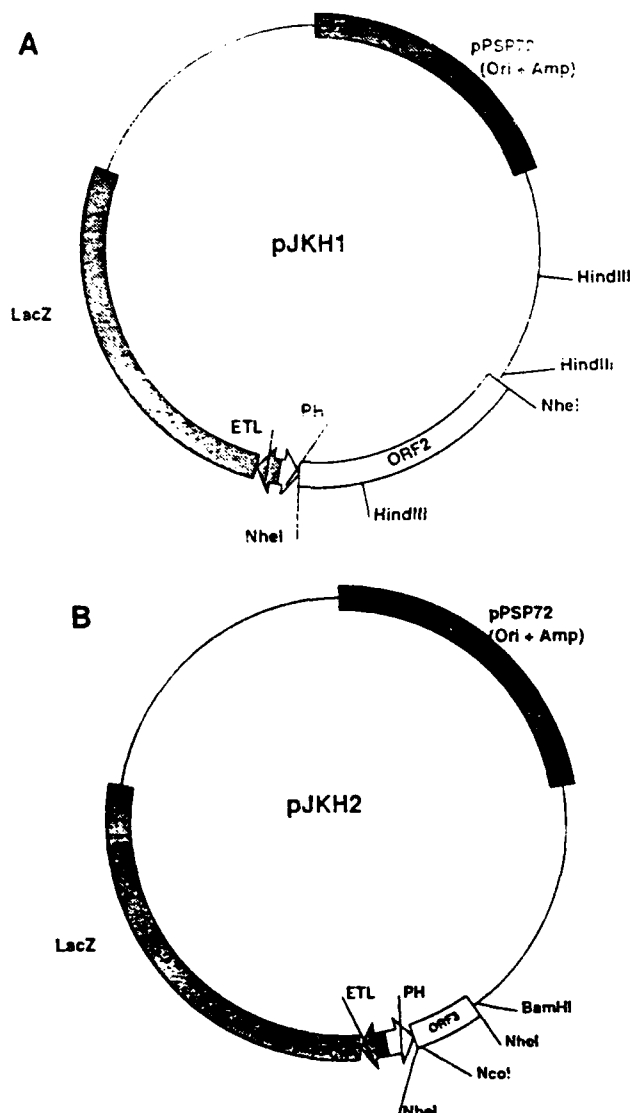


FIG. 1. Graphical representations of the ORF2 and ORF3 PCR products with flanking *Xba*I ends ligated into the *Nhe*I site of pBlue-Bac2, resulting in pJKH1 (A) and pJKH2 (B), respectively.

phosphatase-labeled anti-goat IgM immunoglobulin (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) in wash medium for 1 h at room temperature. After an additional wash, the nitrocellulose membranes were then incubated for 15 min with nitroblue tetrazolium (0.1 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate (0.2 mg/ml) in diethanolamine buffer (Kirkegaard & Perry).

**Immunofluorescence.** Uninfected Sf9 cells and Sf9 cells infected with AcNPV or infected with rAcNPV at a multiplicity of infection of approximately 1 were cultured in Lab-Tek Flaskette Biological Growth Chambers (Miles Scientific, Naperville, Ill.). After 72 h of growth, slides with the adherent Sf9 cells were fixed for 10 min in 50% methanol and 50% acetone at room temperature. Slides were then blocked with PBS containing 1% normal goat serum (NGS) (Pel Freez Biologicals, Rogers, Ark.) for 1 h. After a washing with PBS-1% NGS, slides were incubated with a 1:200 dilution in PBS-1% NGS of high-titered rabbit polyclonal

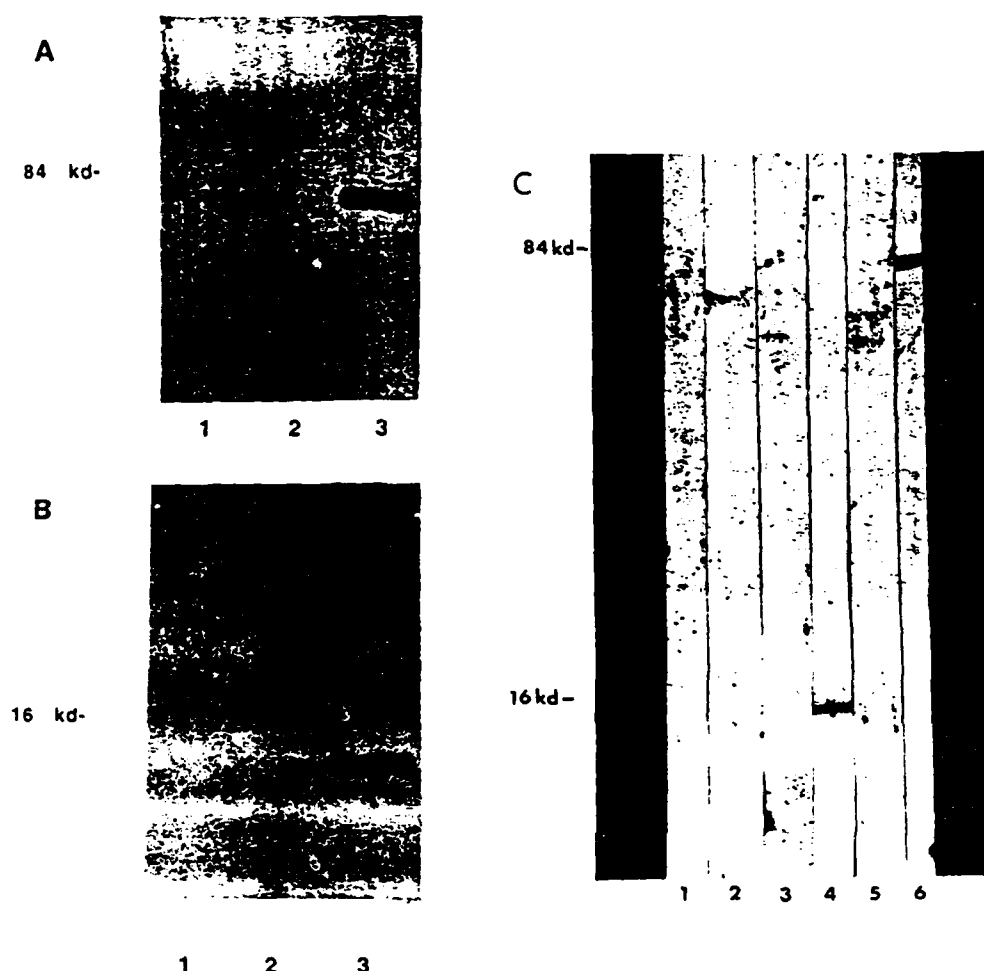


FIG. 2. (A and B) Immunoblot analysis of cell pellet containing uninfected Sf9 cells (lanes 1), Sf9 cells infected with AcNPV (lanes 2), or Sf9 cells infected with ORF2-rAcNPV (A) or ORF3-rAcNPV (B) (lanes 3). Antibody used was a 1:900 dilution of sera from a patient acutely infected with HEV. (C) Immunoblot reactivities of 19 pooled serum samples obtained from control Sudanese patients without acute hepatitis directed against Sf9 cells (lane 1), Sf9 cells infected with wild-type virus (lane 2), Sf9 cells infected with ORF3-rAcNPV (lane 3), or Sf9 cells infected with ORF2-rAcNPV (lane 5). As controls, reactivities of sera obtained from a patient with acute hepatitis E against ORF3-rAcNPV (lane 4) and ORF2-rAcNPV (lane 6) are shown.

sera directed against either the ORF2- or the ORF3-encoded antigens expressed in *E. coli* (unpublished data) or the corresponding preimmune sera. After incubation for 1 h, the slides were washed again with PBS-1% NGS and incubated for 1 h with a 1:60 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG F(ab')<sub>2</sub> (Sigma Immuno Chemicals, St. Louis, Mo.) in PBS-1% NGS. After a washing with PBS-1% NGS, the slides were examined by fluorescence microscopy.

## RESULTS

**Construction of recombinant transfer plasmids.** The PCR-generated ORF2 and ORF3 fragments which included flanking *Xba*I restriction sites were digested with *Xba*I, and the resulting 1,988-bp ORF2 fragment and the 378-bp ORF3 fragment were each ligated into the compatible *Nhe*I site in the transfer vector pBlue-Bac2 which had been previously digested with *Nhe*I and treated with alkaline phosphatase (Fig. 1). Correct orientation of ORF2 and ORF3 fragments in the transfer vector were confirmed by restriction enzyme analysis. pJKH1, a recombinant plasmid containing the

ORF2 fragment, was digested with *Hind*III, and the correct orientation of the fragment within the plasmid was confirmed by the presence of 928-, 1,512-, and 9,880-bp fragments (27) (data not shown). Similarly, pJKH2, a recombinant plasmid containing the ORF3 fragment, was digested with *Bam*HI and *Nco*I, and the correct orientation of the fragment within the plasmid was confirmed by the presence of 350- and 10,301-bp fragments (27) (data not shown).

**Expression of ORF2- and ORF3-encoded proteins in Sf9 cells.** Recombinant baculovirus virions (rAcNPV) were obtained by cotransfection of Sf9 cells with either pJKH1 or pJKH2 and wild-type AcNPV DNA as previously described (14). Recombinant plaques were selected and expanded for 48 h in  $2 \times 10^6$  Sf9 cells, and aliquots of the cell pellets and supernatants were analyzed by Western blot assay using pooled polyclonal human sera previously shown to contain IgM antibodies against a truncated ORF2-*trpE* fusion protein (9). As shown in Fig. 2, an rAcNPV (ORF2-rAcNPV) expressing an ORF2 protein with a calculated molecular weight (based on amino acid sequence) of approximately 70,900 and an rAcNPV (ORF3-rAcNPV) expressing an

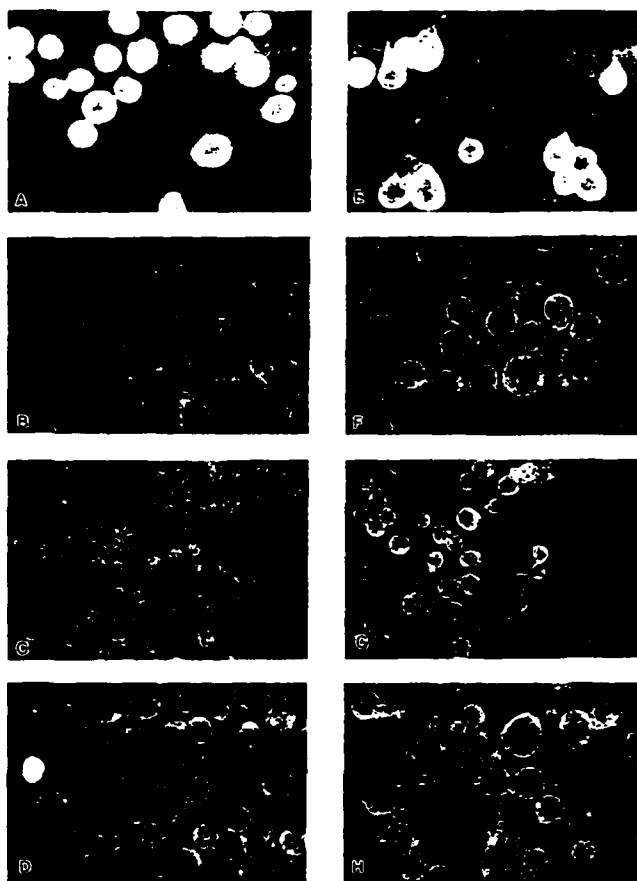


FIG. 3. Analysis of uninfected, ORF2-rAcNPV-infected, and ORF3-rAcNPV-infected Sf9 cells by using immunofluorescence. (A) ORF2-rAcNPV-infected cells incubated with ORF2-specific polyclonal rabbit sera; (B) ORF2-rAcNPV-infected cells incubated with preimmune polyclonal rabbit sera; (C) uninfected cells incubated with ORF2-specific polyclonal rabbit sera; (D) AcNPV-infected cells incubated with ORF2-specific polyclonal rabbit sera; (E) ORF3-rAcNPV-infected cells incubated with ORF3-specific polyclonal rabbit sera; (F) ORF3-rAcNPV-infected cells incubated with preimmune polyclonal rabbit sera; (G) uninfected cells incubated with ORF3-specific polyclonal rabbit sera; (H) AcNPV-infected cells incubated with ORF3-specific polyclonal rabbit sera.

ORF3 protein with a calculated molecular weight (based on amino acid sequence) of 12,700 (ORF3) were observed in cell pellets. No recombinant proteins were identified in cell supernatants (data not shown). Maximal expression of ORF2 and ORF3 recombinant proteins as determined by Western blot analysis occurred 48 and 96 h postinfection, respectively. Sera without detectable anti-HEV obtained from patients in the Sudan suffering from illnesses other than acute hepatitis E were pooled. These pooled sera failed to recognize baculovirus-expressed ORF2 and ORF3 proteins, uninfected Sf9 cells, or Sf9 cells infected with wild-type baculovirus (Fig. 2C). Fifteen additional negative control serum samples obtained from the Philippines failed to react with baculovirus-expressed recombinant ORF2 and ORF3 proteins (data not shown).

**Analysis of rAcNPV-infected cells using an immunofluorescence assay.** Uninfected Sf9 cells, and Sf9 cells infected with AcNPV, ORF2-rAcNPV, or ORF3-rAcNPV at a multiplicity of infection of approximately 1 were examined by using an

immunofluorescence assay with rabbit polyclonal sera specific for ORF2 or ORF3 as well as the corresponding preimmune sera. As shown in Fig. 3, significant cytoplasmic immunofluorescence was observed when Sf9 cells infected with ORF2-rAcNPV were incubated with ORF2-specific polyclonal sera. No such immunofluorescence was observed when the ORF2-rAcNPV-infected cells were incubated with preimmune ORF2 sera or when uninfected or AcNPV-infected Sf9 cells were incubated with ORF2-specific sera. Similarly, significant cytoplasmic immunofluorescence was also observed when Sf9 cells infected with ORF3-rAcNPV were incubated with ORF3-specific polyclonal sera (Fig. 3). By comparison, no such immunofluorescence was observed when the ORF3-rAcNPV-infected cells were incubated with preimmune ORF3 sera or when uninfected or AcNPV-infected Sf9 cells were incubated with ORF3-specific sera.

**Use of ORF2- and ORF3-encoded proteins in Western blot assays.** The recombinant baculovirus-expressed ORF2 and ORF3 proteins were used as antigens in a diagnostic Western blot assay. Sera were obtained from 28 Sudanese pediatric patients with acute hepatitis as well as from 19 Sudanese pediatric control patients who presented with illnesses other than hepatitis. Immunoreactivity of sera from the acute hepatitis patients with the ORF2 baculovirus-expressed recombinant as determined by Western blot assay is shown in Fig. 4A. Serum samples identified as having IgM antibodies against the baculovirus-expressed ORF2 protein were obtained from 17 of 18 patients (94.4%) with NANB hepatitis (Fig. 4A, lanes 9 to 20, 23 to 25, 27, and 28), 3 of 7 patients (42.9%) with acute hepatitis A (lanes 1, 2, and 8), 2 of 2 patients with chronic hepatitis B (lanes 21 and 22) and 1 patient with acute hepatitis B (lane 7). None of the 19 control serum samples contained IgM antibodies which reacted with the ORF2 baculovirus-expressed protein (Fig. 4B). Of the 18 acute hepatitis patients, 10 patients (55.6%) with NANB hepatitis were identified by immunoblot analysis as having IgM antibodies against the baculovirus-expressed ORF3 protein (Fig. 4C, lanes 9, 11, 12, 16, 19, 20, 22, 23, 25, and 27). Sera from all 10 of these patients also reacted with the ORF2 baculovirus protein as described above (Fig. 4A, lanes 9, 11, 12, 16, 19, 20, 22, 23, 25, and 27). None of the 19 control serum samples contained IgM antibodies which reacted with ORF3 baculovirus-expressed protein (Fig. 4D).

## DISCUSSION

In the present study, we have chosen to express the putative structural proteins of HEV in baculovirus. According to a recent review (14), 207 recombinant proteins derived from viruses, bacteria, fungi, plants, and animals have been reported to have been expressed in baculovirus. The advantage of using baculovirus for expression is evident—proteins expressed in insect cells are processed, in most cases, as they would be in mammalian cells, with a resulting authentic, functional protein. Although the limited growth of HEV in a cell culture system has been recently reported (8), quantities of natural HEV proteins sufficient for further studies are not readily available, and it is therefore not known at the present time whether the baculovirus-expressed proteins reported herein are identical to those expressed by HEV.

The Western blot assay using the baculovirus-expressed ORF2 protein described in this article appears to be sensitive and specific for the detection of anti-HEV IgM in human sera. We were able to identify anti-HEV IgM in the sera of 17 of 18 patients with acute NANB hepatitis but not in the

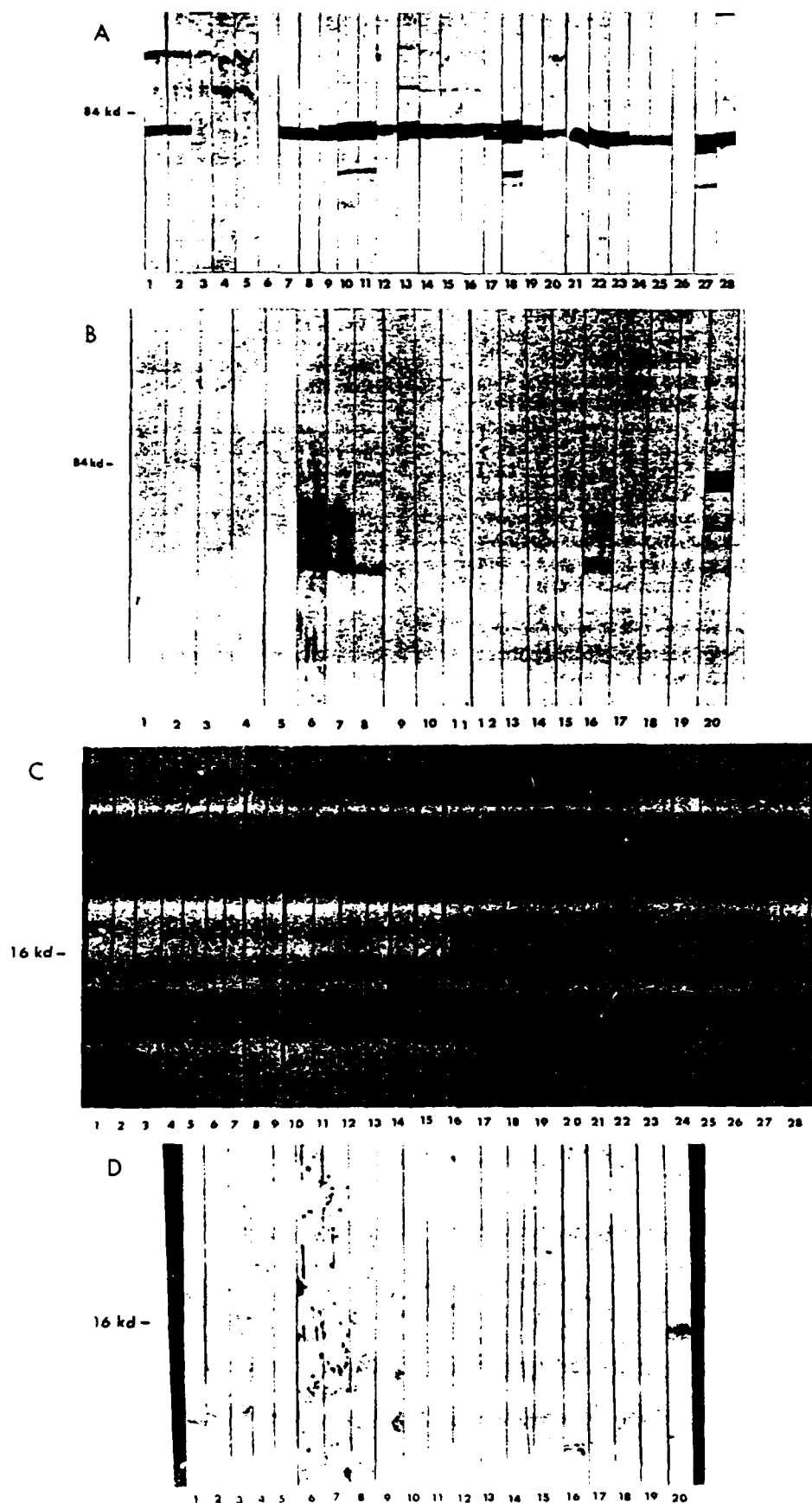


FIG. 4. Immunoblot analysis of sera obtained from 28 (A and C) or 19 (B and D) patients with acute hepatitis. Solubilized Sf9 cells infected with ORF2-rAcNPV (A and B) or ORF3-rAcNPV (C and D) were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and incubated with an optimal concentration (1:900 [A and B] or 1:300 [C and D]) of patient sera prior to colorimetric detection. In panels B and D, immunoblot analysis of serum from a patient with acute hepatitis E is shown in lane 20.

sera of 19 control patients presenting at the same clinic with illnesses other than acute hepatitis. Because of a lack of significant reactivity against Sf9 proteins, we have been able to use crude cell pellets in this assay without significant background reactivities. At present we are unable to account for the IgM anti-HEV antibodies identified in sera from the single patient with IgM anti-hepatitis B core antibody, and it is not clear whether this represents false positivity or simultaneous (or overlapping) infection. It is easier to understand the presence of IgM antibodies against both hepatitis A virus and HEV in the sera of three patients, since simultaneous infection via the fecal-oral route might readily occur.

In the present study, the recombinant ORF2 protein appears to be far more sensitive as a diagnostic antigen than the ORF3 protein. This may be due to several factors. First, although the same amounts of cellular material infected at approximately the same multiplicity of infection with either ORF2-rAcNPV or ORF3-rAcNPV were used in the assays, it is possible that the cellular expression of the ORF2 recombinant protein is better than that of the ORF3 recombinant protein. Therefore, if equimolar quantities of each of these proteins were used in a diagnostic Western blot assay, it is possible that the ORF3 protein might provide the same sensitivity as the ORF2 protein. Alternatively, the increased sensitivity observed with the ORF2 protein may be due to the presence of many epitopes on the ORF2 protein which are recognized by antibodies from acutely infected patients, in contrast to the single epitope which is located at the carboxy terminus of the ORF3 protein (11).

Cloned PCR products used in the present study to generate the recombinant baculovirus-expressed ORF2 and ORF3 proteins were derived from the HEV Burma strain (21). Since there appears to be some divergence in the nucleotide sequences of HEV isolates from different geographical regions (21, 29), there may also be differential antibody responses against these isolates. In support of this hypothesis is our preliminary observation that sera derived from patients acutely infected with HEV in Cairo do not react as strongly with the baculovirus-expressed ORF2 protein as do sera from acutely infected patients from the Sudan (data not shown). We are presently using this Burma strain-derived ORF2 protein to screen sera from patients with acute NANB hepatitis from other geographical locations to determine whether this antigen can be universally used for the diagnosis of hepatitis E or whether it will be necessary to express recombinant ORF2 proteins derived from other isolates.

#### ACKNOWLEDGMENTS

This investigation was supported by the Naval Medical Research and Development Command, Department of the Navy, Research Task Number 3M162787A870 AQ208.

We thank Craig Hyams for supplying patient sera, Stephanie Grey and William Jones for technical assistance, and Y. H. Kang and Rob Williams for help in preparation of photographs.

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